

FORM-PTO-1390 (Rev. 10-96)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 001560-344
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unassigned <b>09/254152</b>
INTERNATIONAL APPLICATION NO. PCT/JP97/02989	INTERNATIONAL FILING DATE 27 August 1997 ✓	PRIORITY DATE CLAIMED 30 August 1996 ✓	
TITLE OF INVENTION PROCESS FOR PRODUCING UNSATURATED FATTY ACID-CONTAINING OILS ✓			
APPLICANT(S) FOR DO/EO/US Kenichi HIGASHIYAMA; Kengo AKIMOTO; and Sakayu SHIMIZU. ✓			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). <b>Items 11. to 16. below concern other document(s) or information included:</b> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input checked="" type="checkbox"/> Other items or information: PCT FORM RO/101; Amendment under Article 34; PCT Form 308; Pct Form 332; and International Search Report.			

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.50) <b>Unassigned</b>		INTERNATIONAL APPLICATION NO. <b>PCT/JP97/02989</b>		ATTORNEY'S DOCKET NUMBER <b>001560-344</b>	
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17. <input checked="" type="checkbox"/> The following fees are submitted:				<b>CALCULATIONS</b>	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$840.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... \$670.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... \$760.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$96.00  <div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>					
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	29 -20 =	9	X\$18.00	\$ 162.00	
Independent Claims	5 -3 =	2	X\$78.00	\$ 156.00	
Multiple dependent claim(s) (if applicable)			+ \$260.00	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 1,158.00	
Reduction for 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$	
<b>SUBTOTAL =</b>				\$ 1,158.00	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30				\$	
<b>TOTAL NATIONAL FEE =</b>				\$ 1,158.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$ 40.00	
<b>TOTAL FEES ENCLOSED =</b>				\$ 1,198.00	
				Amount to be: refunded	\$
				charged	\$

  

a. ☒ A check in the amount of \$ 1,198.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 02-4800 in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

Ronald L. Grudziecki  
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SIGNATURE  
  
 NAME  
  
 30,888  
 REGISTRATION NUMBER

February 26, 1999

09/254152  
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Patent  
Attorney's Docket No. 001560-344

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
Kenichi HIGASHIYAMA et al.	)	Group Art Unit: Unassigned
Application No.: Unassigned	)	Examiner: Unassigned
Filed: February 26, 1999	)	
For: PROCESS FOR PRODUCING	)	
UNSATURATED FATTY	)	
ACID-CONTAINING OILS	)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified patent application please amend the application as follows:

IN THE CLAIMS:

*The Examiner is respectfully requested to examine the claims as amended in the Article 34 amendment filed in the PCT International application and as amended below.*

Please amend claims 4, 5, 6, 7, 9, 10, 11, 15, 16, 17, 18, 21, 23, 24, 25, 26, 27, and 28 as follows:

Claim 4, line 2, delete "or 2".

Claim 5, line 2, delete "or 2".

Claim 6, line 2, delete "any one claims 1, 2, 4, and 5" to --claim 1--.

Claim 7, line 2, change "any one of claims 1, 2 and 4 to 6" to --claim 1--.

Claim 9, line 2, delete "or 8".

Claim 10, lines 1 and 2, change "any one of claims 7 to 9" to --claim 7--.

Claim 11, line 2, change "any one of claims 1, 2 and 4 to 10" to --claim 1--.

Claim 15, line 2, delete "or 14".

Claim 16, line 3, change "any one of claims 13 to 15" to --claim 13--.

Claim 17, lines 3 and 4, change "any one of claims 13 to 15" to --claim 13--.

Claim 18, lines 2 and 3, change "any one of claims 13 to 15" to --claim 13--.

Claim 21, line 2, delete "or 20".

Claim 23, line 2, delete "or 20".

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Attorney's Docket No. 001560-344

Claim 24, line 2, change "any one of claims 19 to 23" to  
--claim 19--.

Claim 25, line 2, change "any one of claims 19 to 24" to  
--claim 19--.

Claim 26, line 2, change "any one of claims 19 to 25" to  
--claim 19--.

Claim 27, line 2, change "any one of claims 19 to 26" to  
--claim 19--.

Claim 28, line 2, change "any one of claims 19 to 27" to  
--claim 19--.

REMARKS

In the event that there are any questions concerning this amendment, or the application in general, the Examiner is respectfully urged to telephone the undersigned attorney so that prosecution of the application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

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## DESCRIPTION

PROCESS FOR PRODUCING UNSATURATED FATTY  
ACID-CONTAINING OILS

5 Field of the Invention

The present invention relates to a process for producing unsaturated fatty acid-containing oils with a low 24,25-methylencholest-5-en-3 $\beta$ -ol content using microorganisms belonging to the genus *Mortierella*,  
10 subgenus *Mortierella*.

Related Art

Microorganisms belonging to the genus *Mortierella*, subgenus *Mortierella* are known as microorganisms which produce unsaturated fatty acids such as arachidonic acid,  
15 dihomog- $\gamma$ -linolenic acid and eicosapentaenoic acid, and processes have been developed for efficient production of arachidonic acid, dihomog- $\gamma$ -linolenic acid and eicosapentaenoic acid by fermentation using these microorganisms (Japanese Unexamined Patent Publications  
20 No. 63-44891, No. 63-12290, No. 63-14696, No. 5-91887, No. 63-14697). In addition there is also known a process for producing Mead acid using mutant strains having reduced or defective in  $\Delta 12$  desaturating activity, which are obtained by mutating microorganisms belonging to the  
25 genus *Mortierella*, subgenus *Mortierella* (Japanese Unexamined Patent Publication No. 5-91888).

Unsaturated fatty acids such as dihomog- $\gamma$ -linolenic acid, arachidonic acid, eicosapentaenoic acid and Mead acid are precursors of prostaglandins, thromboxanes,  
30 prostacyclins, leucotrienes and the like which have powerful and versatile physiological activity, and much attention is therefore being directed to foods and animal feeds to which these are added.

For example, arachidonic acid is said to be a  
35 precursor of prostaglandins, thromboxanes, prostacyclins and leucotrienes which exhibit physiological activity including uterine muscle contracting and relaxing

effects, vasodilator and antihypertensive effects, etc., and recently research has been rapidly progressing on docosahexaenoic acid (hereunder also abbreviated to "DHA") as an essential component particularly for infant development.

Specifically, Lanting et al. (LANCET, Vol.344, 1319-1322(1994)) have examined infants raised on breast milk and infants raised on infant powdered milk for 3 weeks or more after birth, with follow-up to 9 years of age, studying the incidence of minor damage to cranial nerves from a behavioral perspective, and have reported that the incidence of brain damage in children raised on infant powdered milk is twice that of children raised on breast milk. This shocking result suggests that higher unsaturated fatty acids such as DHA and arachidonic acid which are present in breast milk but virtually absent in infant powdered milk play a role in the development of the brain. Subsequent reports have also shown results suggesting that higher unsaturated fatty acids are connected with the development of the brain and retina.

Nevertheless, while unsaturated fatty acid-containing oils are considered to be highly safe, the issue of their microbial sources has prevented them from wide use throughout the world; meanwhile, in LIPIDS, Vol.27, No.6, 481-483(1992), *Mortierella alpina* 1S-4 was reported to produce 24,25-methylencholest-5-en-3 $\beta$ -ol which to that time was not known to occur naturally. Thus, it has been desired to develop unsaturated fatty acid-containing oils obtained from microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* which can be more safely utilized for foods and animal feeds.

#### Disclosure of the Invention

It is therefore an object of the present invention to provide a microorganic oil which can be safely used in foods and animal feeds and which can economically and stably provide unsaturated fatty acids.

In order to solve the problem described above, the present inventors have searched for a process for efficient production of unsaturated fatty acid oils with a low content of 24,25-methylencholest-5-en-3 $\beta$ -ol the use of which as a food is still unknown, and have studied in detail the relationship between various medium components and sterol compositions; as a result they have completed the present invention upon finding that it is possible to obtain oils with a low compositional ratio of 24,25-methylencholest-5-en-3 $\beta$ -ol by using a nitrogen source derived from soybean for culturing of microorganisms belonging to the genus *Mortierella* subgenus *Mortierella*.

In other words, the present invention relates to a process for producing unsaturated fatty acid-containing oils, which comprises culturing a microorganism belonging to the genus *Mortierella* subgenus *Mortierella* in a medium containing a nitrogen source derived from soybean, and collecting the unsaturated fatty acid-containing oil from the culture product.

#### Embodiment for Carrying Out the Invention

According to the invention, unsaturated fatty acids are fatty acids with at least 16 carbon atoms and at least one double bond, among which higher unsaturated fatty acids are generally fatty acids with at least 18 carbon atoms and at least two double bonds, and as examples there may be mentioned  $\gamma$ -linolenic acid, dihomo- $\gamma$ -linolenic acid, arachidonic acid, eicosapentaenoic acid and Mead acid.

As examples of microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* according to the invention there may be mentioned *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila*, *Mortierella alpina*, etc., and specifically there may be mentioned *Mortierella elongata* IF08570, *Mortierella exigua* IF08571, *Mortierella hygrophila* IF05941, *Mortierella alpina* IF08568, ATCC16266, ATCC32221, ATCC42430, CBS219.35,



CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS529.72, CBS608.70, CBS754.68 and other cell lines.

These strains are all obtainable without restrictions from the Institute of Fermentation, Osaka (IFO), American Type Culture Collection (ATCC) and Centraalbureau voor Schimmelcultures (CBS). *Mortierella elongata* SAM0219 (FERM P-8703) (FERM-BP 1239) which was isolated from soil by the research group for the present invention, may also be used. These type culture cell lines or naturally occurring isolated cell lines may be used directly, but by growth and/or isolation at least once it is possible to obtain a natural mutant with different properties than the original cell line.

The microorganisms used according to the invention include mutant strains or recombinant strains of microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* (wild strains), i.e. those designed either to give a higher unsaturated fatty acid content in the oil, a higher total oil content, or both, compared to the amount produced by the original wild strain, when cultured using the same substrate.

Also included are microorganisms designed to produce the same amount of unsaturated fatty acid as the wild strain through the efficient use of a substrate with an excellent cost effect. As examples there may be mentioned *Mortierella alpina* SAM1861 (FERM BP-3590) as a mutant strain defective in  $\Delta 12$  desaturating activity and *Mortierella alpina* SAM1860 (FERM BP-3589) as a mutant strain defective in  $\Delta 5$  desaturating activity.

The above-mentioned microorganisms belonging to the genus *Mortierella* subgenus *Mortierella* in the form of spores, hypha or a preculture obtained by previous culturing are inoculated into a liquid medium or solid medium and cultured. The carbon source used may be glucose, fructose, xylose, saccharose, maltose, soluble starch, molasses, glycerol, mannitol, citric acid, corn starch or any other conventional one, but glucose,

maltose, fructose, corn starch, glycerol and citric acid are particularly preferred.

According to the invention, by using a nutrient source obtained from soybean as the nitrogen source it is possible to lower the compositional ratio of 24,25-methylencholest-5-en-3 $\beta$ -ol in the oil.

The soybean-derived nitrogen source used for the invention is one with a nitrogen content of at least 2 wt%, preferably at least 3 wt% and more preferably at least 5% with respect to the total components except for water. The soybean-derived nitrogen source may be one or a combination of different types of defatted soybean or soybean subjected to heat treatment; acid treatment; alkali treatment; enzyme treatment; chemical modification; denaturation and/or renaturation by chemical and/or physical processing including heat treatment, acid treatment, alkali treatment, enzyme treatment, chemical modification, etc.; removal of a portion of the components with water and/or organic solvents; removal of a portion of the components by filtration and/or centrifugation; freezing; crushing; drying; sifting; etc., or a product of processing in the same manner as non-defatted soybean; as common candidates there may be mentioned soybean, defatted soybean, soybean flakes, edible soybean protein, okara, soy milk and roasted soybean flour (kinako), among which are particularly preferred heat-denatured defatted soybean, and especially heat-denatured defatted soybean from which the ethanol-soluble components have been further removed.

When necessary one or more different additional nitrogen sources may also be added so long as the sterol composition is not notably affected, and examples include organic nitrogen sources such as peptone, yeast extract, malt extract, meat extract, casaminic acid, corn steep liquor and urea, and inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and ammonium sulfate.

Also, when necessary trace nutrient sources may be

used, including inorganic salts such as potassium phosphate, potassium dihydrogen phosphate and other phosphate salts, ammonium sulfate, sodium sulfate, magnesium sulfate, iron sulfate, copper sulfate, magnesium chloride, calcium chloride, etc., and vitamins.

According to the invention, accumulation of the unsaturated fatty acid of interest may be accelerated by accomplishing the culturing with addition of a substrate for the unsaturated fatty acid in the medium. The unsaturated fatty acid substrate used may be, for example, a hydrocarbon such as hexadecane or octadecane; a fatty acid such as oleic acid or linolic acid or a salt, for example a sodium or potassium salt thereof, or a fatty acid ester such as an ethyl ester, glycerol fatty acid ester or sorbitan fatty acid ester; or an oil such as olive oil, soybean oil, rapeseed oil, cottonseed oil or coconut oil, and these may be used alone or in combinations. The total amount of the substrate added is 0.001 to 10 wt%, and preferably 0.5 to 10 wt%, with respect to the medium. Any of these substrates may also be used as the sole carbon source for culturing.

The above-mentioned carbon sources, nitrogen sources, inorganic salts, vitamins and/or additives may be added to the medium prior to the start of culturing or to the culture broth during the cultivation. The medium components can be added all at once, or continuously or periodically through a few additions. The medium components may each be added alone or as a mixture. There are no particular restrictions on the concentrations of the medium components so long as growth of the cells is not inhibited. In practical usage, the carbon source should be at a concentration of 0.1 to 30 wt%, preferably 1 to 15 wt%, and the nitrogen source should be at a concentration of 0.01 to 10 wt%, and preferably 0.1 to 5 wt%.

The culturing temperature is 5 to 40°C, and preferably 20 to 30°C, and the unsaturated fatty acid may

also be produced by growth of the cells by culturing at 20 to 30°C followed by continued culturing at 5 to 20°C. This manner of temperature control may also be employed to increase the yield of higher unsaturated fatty acids content in the fatty acids which are produced. The pH of the medium is 4 to 10, and preferably 5 to 8, and culturing with aeration and agitation, shake culturing or static culturing may be employed. The culturing is normally carried out for 2 to 20 days, preferably 5 to 20 days, and more preferably 5 to 15 days.

A fermenter, especially culturing fermenter with aeration and agitation or air-lift culturing fermenter may be used for submerged culturing with aeration to enable production with yields suited for unsaturated fatty acid-containing oils as commercial products. In such cases, the unsaturated fatty acid-containing oil can be even more efficiently produced by maintenance during the culturing to a glucose concentration of at least 0.3 wt% and/or an average glucose concentration of at least 0.5 wt%, preferably a glucose concentration of at least 0.5 wt% and/or an average glucose concentration of at least 0.7 wt%, and more preferably a glucose concentration of 0.5-5 wt% and/or an average glucose concentration of 0.7-3 wt%, for at least 3 days after the start of culturing. For example, arachidonic acid can be produced at 100 mg or more, and preferably 120 mg or more to one gram of dry cells.

Thus, an oil which is rich in the desired unsaturated fatty acid and low in 24,25-methylenecholest-5-en-3 $\beta$ -ol, is accumulated in large quantities in the cells.

The desired oil can be obtained according to a conventional method from the culture broth taken during production of the oil by the cell culturing or after its sterilization, the culture broth obtained at the end of culturing or after its sterilization, or the cultured cells collected from either, alternatively in dry form.

The desired oil may be collected from the cultured cells by the following method, for example.

After culturing is complete, the cultured cells are obtained from the culture broth by a conventional  
5 solid/liquid separation means such as centrifugation and/or filtration. The cultured cells are preferably washed, disrupted and dried. The drying may be accomplished by freeze drying, air drying or the like. The dry cells are preferably subjected to extraction with  
10 an organic solvent preferably under a nitrogen stream. The organic solvent used may be ether, hexane, methanol, ethanol, chloroform, dichloromethane, petroleum ether or the like, and satisfactory results can also be obtained by alternate extraction with methanol and petroleum ether  
15 or by extraction using a chloroform-methanol-water monolayer system.

By removing the organic solvent from the extract under reduced pressure, it is possible to obtain an unsaturated fatty acid-containing oil at a high  
20 concentration. The extraction may also be accomplished using wet cells, instead of by the method described above. In this case there is used a water-compatible solvent such as methanol or ethanol, or a water-compatible mixed solvent including one of these with  
25 water and/or another solvent. The other procedures are the same as described above.

The oil obtained in this manner contains the unsaturated fatty acids in a state of triglycerides and phosphatidylcholine, phosphatidylethanolamine or  
30 phosphatidylinositol, but most of it is in the form of triglycerides. In order to separate and purify the unsaturated fatty acid-containing triglycerides from the unsaturated fatty acid-containing oil collected from the cultured product, a conventional method may be used for  
35 hexane extraction followed by deacidification, decoloration, deodorization and degumming treatment, or cooling separation.

According to the invention, the compositional ratio of 24,25-methylenecholest-5-en-3 $\beta$ -ol is determined by the following method based on sterol composition analysis.

The sterol composition analysis will be explained first. A 30 to 80 mg portion of the oil is weighed out into a test tube with stopper, 4 mL of methanol and 1 mL of a 33% aqueous potassium hydroxide solution are added, and the stopper is fitted. After reaction for one hour while gently stirring at 80°C, the mixture is allowed to be cooled and the oil-soluble components are extracted with hexane. The resulting hexane solution is washed with water until a phenolphthalein indicator does not color the aqueous layer, and is then concentrated under a reduced pressure to obtain an analytical sample. The analytical sample is dissolved in a small amount of hexane and subjected to gas chromatography under the conditions listed in the table given below. By comparing the gas chromatogram with a commercially available desmosterol standard, the desmosterol peaks are identified.

The components which are detected within 0.8 to 2.0 times the retention time of desmosterol are the sterol components, and the peak areas of the gas chromatograms for all of the sterol components within the retention time are determined by a conventional method. The ratio of the peak area of each component to the sum of the total peak areas of the components is taken as the compositional ratio of each component. For example, the ratio of the peak area detected for desmosterol with respect to the sum of the total sterol area is the compositional ratio of desmosterol. 24,25-methylenecholest-5-en-3 $\beta$ -ol is detected in a retention time of 1.07 to 1.12 times the retention time of desmosterol. The ratio of the peak area detected for 24,25-methylenecholest-5-en-3 $\beta$ -ol with respect to the sum of all the peak areas is the compositional ratio of 24,25-methylenecholest-5-en-3 $\beta$ -ol.

Column used: ULBON HR-1 (inner diameter: 0.25 mm,  
length: 25 m)

Column temperature: 280°C

Inlet and detector temperature: 300°C

5 Carrier gas and gauge pressure, helium: 1.2 kg/cm<sup>2</sup>

Make-up gas and flow rate, nitrogen: 70 mL/minute

Detector: FID

Split ratio: 20

10 The unsaturated fatty acid-containing oil of the  
invention has a 24,25-methylenecholest-5-en-3β-ol  
compositional ratio of 35% or less, preferably 33% or  
less, and more preferably 30% or less, and/or the 24,25-  
methylenecholest-5-en-3β-ol proportion is 1.2 or lower,  
preferably 0.9 or lower and more preferably 0.6 or lower  
15 with respect to the desmosterol present in the oil.  
Desmosterol is a component included with 24,25-  
methylenecholest-5-en-3β-ol in oils obtained by culturing  
microorganisms belonging to the genus *Mortierella*  
subgenus *Mortierella*, and it is known to be present in  
20 breast milk.

As an example of an unsaturated fatty acid-  
containing oil according to the invention there may be  
mentioned an arachidonic acid-containing oil with 20 to  
54 wt% and preferably 30 to 50 wt% arachidonic acid with  
25 respect to the total fatty acids in the oil, and a 24,25-  
methylenecholest-5-en-3β-ol compositional ratio of 35% or  
lower, preferably 33% or lower and more preferably 30% or  
lower and/or a 24,25-methylenecholest-5-en-3β-ol  
proportion of 1.2 or lower, preferably 0.9 or lower and  
30 more preferably 0.6 or lower with respect to the  
desmosterol present in the oil.

The oil properties of the arachidonic acid-  
containing oil are such that the triglyceride content is  
90% or greater, the moisture content is 0.1% or lower,  
35 the acid value is 0.5 or lower and the peroxide value is  
5 or lower, while the color is ≤50 yellow and ≤10 by the

Lovibond method in a 133.4 mm cell, and the fatty acid composition is 20 to 54%, with preferably 30 to 50% arachidonic acid, 0.2 to 0.7% myristic acid, 10 to 16% palmitic acid, 4 to 10% stearic acid, 5 to 15% oleic acid, 5 to 15% linolic acid, 1 to 5%  $\gamma$ -linolenic acid, 0.1 to 2%  $\alpha$ -linolenic acid, 1 to 6% dihomog $\gamma$ -linolenic acid, 0 to 1% eicosapentenoic acid and 2 to 7% lignoceric acid.

The oil is rich in the triglyceride form of arachidonic acid, and either contains no eicosapentenoic acid or contains it only in a very trace amount, and is therefore desirable as a material for foods, and especially immature infant formula, infant formula, baby food and pregnancy food. The unsaturated fatty acid-containing oil of the invention can also be safely used in foods and animal feeds because of its low content of 24,25-methylencholest-5-en-3 $\beta$ -ol, the edibility of which has not yet been established.

#### Examples

The present invention will now be explained in more detail by way of examples.

#### Example 1

Using *Mortierella elongata* IF08570 as the arachidonic acid-producing cell line, 1400 L of a medium containing 2% glucose, 1% edible soybean protein (trade name: Esusan Meat, product of Ajinomoto Co.) and 0.1% rapeseed oil was placed in a 2000 L fermenter equipped with an agitator and aerator and culturing with aeration and agitation was initiated under conditions of 28°C temperature, 1.0 vvm aeration, 80 rpm agitation and 1.0 kg/cm<sup>2</sup>G headspace pressure. The glucose concentration was kept at 1.5% by feeding glucose, and after culturing for 7 days the cells were recovered by filtration and subjected to oil extraction. As a comparative example, culturing and oil extraction were carried out in the same manner using 1% yeast extract instead of the edible



soybean protein.

Upon analyzing the sterol composition of the resulting oil according to the procedure described above, desmosterol was detected at a retention time of about 9.6 minutes and 24,25-methylenecholest-5-en-3 $\beta$ -ol was detected at a retention time of about 10.5 minutes. In the comparative example, desmosterol was detected at a retention time of about 6.5 minutes and 24,25-methylenecholest-5-en-3 $\beta$ -ol was detected at a retention time of about 7.2 minutes. The results are shown in Table 1. Thus, an arachidonic acid-containing oil was obtained with a low compositional ratio of 24,25-methylenecholest-5-en-3 $\beta$ -ol.

Table 1

	24,25-methylene cholest-5-en-3 $\beta$ -ol compositional ratio (A)	Desmosterol compositional ratio (B)	A/B	Total sterol content*	Arachidonic acid content**
Example	30%	65%	0.46	1%	8%
Comp. Example	65%	27%	2.41	1%	9%

\* Sterol content in oil

\*\* Arachidonic acid content with respect to total fatty acids in oil

#### Example 2

*Mortierella alpina* CBS754.68 was used as the arachidonic acid-producing cell line, and 600 L of a medium containing 4% glucose, 1.3% roasted soybean flour (kinako), 0.2% yeast extract and 0.1% olive oil was placed in a 1000 L fermenter equipped with an agitator and aerator, for culturing with aeration and agitation for 5 days under conditions of 24°C temperature, 1.0 vvm aeration, 100 rpm agitation and 0.5 kg/cm<sup>2</sup>G headspace pressure, followed by filtration and drying to recover the cells and hexane extraction to obtain an oil. As a comparative example, culturing was carried out in the same manner using a medium of 4% glucose, 1.5% yeast extract and 0.1% olive oil to obtain an oil. In both the example and the comparative example, 1% glucose was added

on the 2nd day of culturing.

Upon analyzing the sterol composition of the resulting oil according to the procedure described above, desmosterol was detected at a retention time of about 10.2 minutes and 24,25-methylenecholest-5-en-3 $\beta$ -ol was detected at a retention time of about 11.2 minutes. In the comparative example, desmosterol was detected at a retention time of about 6.4 minutes and 24,25-methylenecholest-5-en-3 $\beta$ -ol was detected at a retention time of about 7.1 minutes. The results are shown in Table 2. Thus, an arachidonic acid-containing oil was obtained with a low compositional ratio of 24,25-methylenecholest-5-en-3 $\beta$ -ol.

Table 2

	24,25-methylene cholest-5-en-3 $\beta$ -ol compositional ratio (A)	Desmosterol compositional ratio (B)	A/B	Total sterol content*	Arachidonic acid content**
Example	25%	53%	0.47	1.2%	48%
Comp. Example	68%	16%	4.25	1.1%	46%

\* Sterol content in oil

\*\* Arachidonic acid content with respect to total fatty acids in oil

### Example 3

*Mortierella alpina* ATCC32221 and *Mortierella alpina* ATCC42430 were used as arachidonic acid-producing cell lines, and each was cultured. After placing 25 L of a medium containing 4% glucose, 1.2% defatted soy powder, 0.2% potassium hydrogen phosphate and 0.1% soybean oil in a 50 L fermenter equipped with an agitator and aerator, culturing with aeration and agitation was carried out for 5 days under conditions of 28°C temperature, 1.0 vvm aeration, 300 rpm agitation and 1.0 kg/cm<sup>2</sup>G headspace pressure, followed by filtration and drying to recover the cells and hexane extraction to obtain an oil from the recovered cells.

As a comparative example, culturing was carried out in the same manner using a medium of 4% glucose, 1.2%

beer yeast powder, 0.2% potassium hydrogen phosphate and 0.1% rapeseed oil to obtain an oil. In both the example and the comparative example, 1% glucose was added on the 2nd day of culturing. The sterol composition of the resulting oil was analyzed according to the procedure described above. The results are shown in Table 3. Thus, an arachidonic acid-containing oil was obtained with a low compositional ratio of 24,25-methylenecholest-5-en-3 $\beta$ -ol.

Table 3

	24,25-methylene cholest-5-en-3 $\beta$ -ol compositional ratio (A)	Desmosterol composi- tional ratio (B)	A/B	Total sterol content*	Arachidonic acid content**
<i>Mortierella alpina</i> ATCC32221	5%	67%	0.07	0.9%	25%
Comp. Example	37%	28%	1.32	0.8%	20%
<i>Mortierella alpina</i> ATCC42430	5%	35%	0.14	0.9%	18%
Comp. Example	40%	25%	1.60	1.0%	18%

\* Sterol content in oil

\*\* Arachidonic acid content with respect to total fatty acids in oil

#### Example 4

Using *Mortierella alpina* CBS754.68 as the arachidonic acid-producing cell line, 1400 L of a medium containing 2% glucose, 1.5% soybean protein and 0.1% soybean oil was placed in a 2000 L fermenter equipped with an agitation and aeration, and culturing with aeration and agitation was initiated under conditions of 24°C temperature, 1 vvm aeration, 80 rpm agitation and 200 kPa headspace pressure. The glucose concentration was kept at 0.5 to 1.5% by feeding glucose, and after culturing for 7 days the cells were recovered by filtration. After drying the cells, they were extracted with hexane, the extracted oil was subjected to deacidification, decoloration and deodoration, and 0.05% tocopherol was added as an antioxidant. The resulting

oil was analyzed and found to have the following composition.

Analysis results

Triglyceride content: 95.6%

5 Moisture: 0.04%

Acid value: 0.08

Peroxide value: 2.16

Color (Lovibond method, 133.4 mm cell): yellow: 20.1,  
red: 1.4

10 Fatty acid composition:

arachidonic acid 44.4%

myristic acid 0.6%

palmitic acid 14.6%

stearic acid 8.8%

15 oleic acid 6.3%

linolic acid 10.2%

$\gamma$ -linolenic acid 3.2%

$\alpha$ -linolenic acid 0.8%

dihomo- $\gamma$ -linolenic acid 5.2%

20 eicosapentenoic acid 0.2%

lignoceric acid 4.8%

Total sterol content: 1.0%

24,25-methylenecholest-5-en-3 $\beta$ -ol compositional ratio:  
24%

25 Desmosterol compositional ratio: 67%

Example 5

The arachidonic acid-containing oil obtained in Example 4 was appropriately mixed with fish oil and vegetable oil to obtain an essential fatty acid-adjusted  
30 oil. In addition to the essential fatty acid-adjusted oil, the raw materials and components listed below were prepared for formulation of 100 kg of powdered infant formula. After dissolving, mixing and refining the raw materials according to conventional methods, they were  
35 sterilized, concentrated and homogenized, and then spray dried to obtain powdered infant formula.

Raw materials and components

	casein	5.6 kg
	whey protein concentrate	24.0 kg
	essential fatty acid-adjusted oil	25.0 kg
	(composed mainly of linolic acid, 5 $\alpha$ -linolenic acid)	
	arachidonic acid content	80 g
	docosahexaenoic acid content	25 g
	eicosapentaenoic acid content	10 g
	saccharides (lactose and oligosaccharides)	43.4 kg
10	minerals and vitamins	2 kg
	TOTAL	100 kg

CLAIMS

1. (amended) A process for production of an unsaturated fatty acid-containing oil, characterized by culturing a microorganism belonging to the genus  
5 *Mortierella* subgenus *Mortierella* in a medium containing a nitrogen source derived from defatted soybean or physically or chemically processed defatted soybean, and collecting the unsaturated fatty acid-containing oil from the cultured product.
- 10 2. (amended) A process for production according to claim 1, characterized in that said nitrogen source derived from defatted soybean has a nitrogen content of at least 2 wt% with respect to the total components except for water.
- 15 3. (deleted)
4. (amended) A process for production according to claim 1 or 2, characterized in that the processing of said defatted soybean is heat treatment; acid treatment; alkali treatment; enzyme treatment; chemical  
20 modification; denaturation and/or renaturation by chemical and/or physical processing including said treatment; removal of a portion of the components with water and/or organic solvents; removal of a portion of the components by filtration and/or centrifugation;  
25 freezing; crushing; drying; and/or sifting.
5. (amended) A process for production according to claim 1 or 2, characterized in that said nitrogen source derived from defatted soybean is from defatted soybean subjected at least to heat denaturation.
- 30 6. (amended) A process for production according to any one of claims 1, 2, 4 and 5, characterized in that said cultured product is the culture broth taken during production of the oil by the cell culturing or after its sterilization, the culture broth obtained at the end of  
35 culturing or after its sterilization, or the cultured cells collected from either, alternatively in dry form.

7. (amended) A process for production according to any one of claims 1, 2 and 4 to 6, characterized in that the culturing of said microorganism is submerged culturing with aeration using a fermentor.

5 8. A process for production according to claim 7, characterized in that said fermentor is a fermentor equipped with an agitator and air sparger or an air-lift fermentor.

10 9. A process for production according to claim 7 or 8, characterized in that the culturing is carried out while maintaining a glucose concentration of at least 0.3 wt% and/or an average glucose concentration of at least 0.5 wt%, for at least 3 days after the start of culturing.

15 10. A process for production according to any one of claims 7 to 9, characterized in that the culturing is carried out for a period of 2 to 20 days.

20 11. (amended) A process for production according to any one of claims 1, 2 and 4 to 10, characterized in that said unsaturated fatty acid is  $\gamma$ -linolenic acid, dihomo- $\gamma$ -linolenic acid, arachidonic acid, eicosapentaenoic acid and/or Mead acid.

12. (deleted)

25 13. (amended) An arachidonic acid-containing oil characterized by having a 24,25-methylenecholest-5-en-3 $\beta$ -ol compositional ratio in a proportion of 0.14 or less with respect to the desmosterol compositional ratio, and an arachidonic acid content of 18 to 54%.

30 14. (amended) An arachidonic acid-containing oil characterized by having a 24,25-methylenecholest-5-en-3 $\beta$ -ol compositional ratio of 35% or lower, a 24,25-methylenecholest-5-en-3 $\beta$ -ol compositional ratio in a proportion of 0.14 or less with respect to the desmosterol compositional ratio, and an arachidonic acid  
35 content of 18 to 54%.

15. (amended) An arachidonic acid-containing oil according to claim 13 or 14, characterized by being a microbial oil obtained from a microorganism belonging to the genus *Mortierella* subgenus *Mortierella*.

5 16. (amended) A nutritive dietary supplement comprising an arachidonic acid-containing oil according to any one of claims 13 to 15.

10 17. (amended) An immature infant formula, infant formula, baby food or pregnancy food product comprising an arachidonic acid-containing oil according to any one of claims 13 to 15.

18. (amended) An animal feed comprising an arachidonic acid-containing oil according to any one of claims 13 to 15.

15 19. (added) A process for production of an unsaturated fatty acid-containing oil, characterized by culturing with aeration a microorganism belonging to the genus *Mortierella* subgenus *Mortierella* in a liquid medium containing a nitrogen source derived from soybean in a fermenter, and the unsaturated fatty acid-containing oil is collected from the cultured product.

20 20. (added) A process for production according to claim 19, characterized in that said nitrogen source derived from soybean has a nitrogen content of at least 2 wt% with respect to the total components except for water.

25 21. (added) A process for production according to claim 19 or 20, characterized in that said nitrogen source derived from soybean is at least one selected from the group consisting of defatted soybean, non-defatted soybean and their processed products.

30 22. (added) A process for production according to claim 21, characterized in that the processing of said defatted soybean or non-defatted soybean is heat treatment; acid treatment; alkali treatment; enzyme treatment; chemical modification; denaturation and/or renaturation by chemical and/or physical processing



including said treatment; removal of a portion of the components with water and/or organic solvents; removal of a portion of the components by filtration and/or centrifugation; freezing; crushing; drying; and/or sifting.

23. (added) A process for production according to claim 19 or 20, characterized in that said nitrogen source derived from soybean is from defatted soybean subjected at least to heat denaturation.

24. (added) A process for production according to any one of claims 19 to 23, characterized in that said cultured product is the culture broth taken during production of the oil by the cell culturing or after its sterilization, the culture broth obtained at the end of culturing or after its sterilization, or the cultured cells collected from either, alternatively in dry form.

25. (added) A process for production according to any one of claims 19 to 24, characterized in that said fermentor is a fermentor equipped with agitator and air sparger or an air-lift fermentor.

26. (added) A process for production according to any one of claims 19 to 25, characterized in that the culturing is carried out while maintaining a glucose concentration of at least 0.3 wt% and/or an average glucose concentration of at least 0.5 wt%, for at least 3 days after the start of culturing.

27. (added) A process for production according to any one of claims 19 to 26, characterized in that the culturing is carried out for a period of 2 to 20 days.

28. (added) A process for production according to any one of claims 19 to 27, characterized in that said unsaturated fatty acid is  $\gamma$ -linolenic acid, dihomogamma-linolenic acid, arachidonic acid, eicosapentaenoic acid and/or Mead acid.

29. (added) A nutritive dietary supplement comprising an arachidonic acid-containing oil characterized by having a 24,25-methylenecholest-5-en-3 $\beta$ -ol compositional ratio of 35% or lower and an arachidonic

acid content of 18 to 54%; an arachidonic acid-containing oil characterized by having a 24,25-methylenecholest-5-en-3 $\beta$ -ol compositional ratio in a proportion of 1.2 or less with respect to the desmosterol compositional ratio  
5 and an arachidonic acid content of 18-54%; or an arachidonic acid-containing oil characterized by having a 24,25-methylenecholest-5-en-3 $\beta$ -ol compositional ratio of 35% or lower, a 24,25-methylenecholest-5-en-3 $\beta$ -ol  
10 compositional ratio in a proportion of 1.2 or less with respect to the desmosterol compositional ratio, and an arachidonic acid content of 18 to 54%.

30. (added) An immature infant formula, infant formula, baby food or pregnancy food product comprising an arachidonic acid-containing oil according to claim 29.

15 31. (added) An animal feed comprising an arachidonic acid-containing oil according to claim 29.

SUMMARY

- 5 A process for production of unsaturated fatty acid-containing oils which is characterized by culturing a microorganism belonging to the genus *Mortierella* subgenus *Mortierella* in a medium containing a nitrogen source derived from soybean, and collecting the unsaturated fatty acid-containing oil from the cultured product. The oils are obtained with a low 24,25-methylenecholest-5-en-3 $\beta$ -ol content.

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### Japanese Language Declaration

### 日本語宣言書

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As a below named inventor, I hereby declare that:

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My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者であると（下記の名称が複数の場合）信じています。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR PRODUCING UNSATURATED FATTY

ACID-CONTAINING OILS

上記発明の明細書（下記の欄でx印がついていない場合は、本書に添付）は、

the specification of which is attached hereto unless the following box is checked:

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☒ was filed on August 27, 1997 ✓  
as United States Application Number or  
PCT International Application Number  
PCT/JP97/02989 and was amended on  
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私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

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### Prior Foreign Application(s)

外国での先行出願

8-230210 (Pat. Appln.) - Japan  
(Number) (Country)  
(番号) (国名)

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Priority Not Claimed

優先権主張なし

30/August/1996 ✓  
(Day/Month/Year Filed)  
(出願年月日)

(Day/Month/Year Filed)  
(出願年月日)

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